AD-A131 438 THE BIOPHYSICAL PROPERTIES OF STROMA FREE HEMOGLOBIN AND WHOLE BLOOD MIXTURES(U) MASONIC MEDICAL RESEARCH LAB UTICA NY L C CERNY ET AL. MAR 80 DAMD17-79-C-9047 1/1

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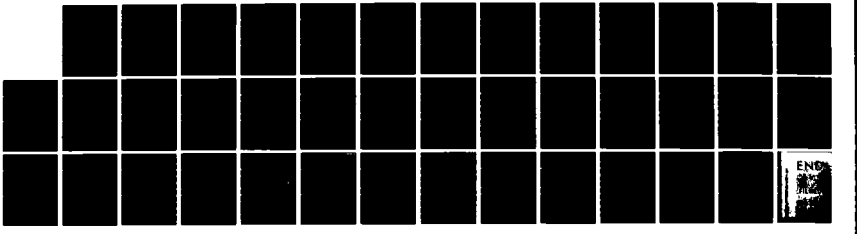

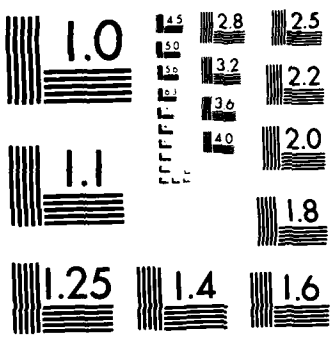

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The Biophysical Properties of Stroma Free Hemoglobin and Whole Blood Mixtures

Annual Progress Report

L. C. Cerny
D. M. Stasiw

March 1980

Supported by

US Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-79-C-9047

Masonic Medical Research Laboratory
Utica, New York 13503

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A131438	3. RECIPIENT'S CATALOG NUMBER
4. TITLE *(and Subtitle)* The Biophysical Properties of Stroma Free Hemoglobin and Whole Blood Mixtures		5. TYPE OF REPORT & PERIOD COVERED Annual Progress Report 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) L. C. Cerny D. M. Stasiw		8. CONTRACT OR GRANT NUMBER(s) DAMD17-79-C-9047
9. PERFORMING ORGANIZATION NAME AND ADDRESS Masonic Medical Research Laboratory Utica, New York 13503		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62772A.3S162772A814.00.045
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command Fort Detrick Frederick, Maryland 21701		12. REPORT DATE March 1980 13. NUMBER OF PAGES 39
14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*		15. SECURITY CLASS. *(of this report)* Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT *(of this Report)* Approved for public release; distribution unlimited		
17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*		
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A

ABSTRACT

Some model studies of the biophysical properties of stroma free hemoglobin and whole blood mixtures have been investigated. The research included the determination of the viscosity, osmotic pressure, the erythrocyte sedimentation rate, the hemolytic malonamide kinetics and osmotic fragility.

These preliminary studies indicate that the concentration of the stroma free hemoglobin plays a critical role in mixtures with whole blood.

Statement of the Problem: This has been a preliminary investigation to establish and to develop the biophysical methodology for the evaluation of the use of stroma-free hemoglobin solutions as blood and plasma substitutes.

Background: Stroma-free hemoglobin solutions are promising plasma substitutes. They have satisfactory oxygen-carrying capacity, do not have any adverse effects on the renal function and offer some rheological advantages. Any ideal plasma substitute should exert a sufficient collodial osmotic pressure to maintain the circulating volume, should not increase the blood viscosity, especially at near stagnant conditions, should aid in the improvment of tissue perfusion and should not interact with the erythrocyte membrane or plasma proteins to cause aggregation. Stroma-free hemoglovin (SFH) meets many of these criteria. However, improvements are always taking place such as the amidination and pyridoxilation of the hemoglobin to increase the oxygen binding capacity, lipid encapsulation of the hemoglobin to prolong its vivo retention, and the addition of suitable polymers to promote consistent rheological behavior. Because of the vast amount of work and interest in SFH and its modifications, it is extremely important to establish a data base through satisfactory model experiments as a means of recognizing and evaluating quantitatively the effects of any changes that may be made.

Approach to the Problem:

Technical Objective (1). The investigation of the flow behavior as a function of hematocrit SFH concentration and the rate of shear.

The viscosity of the stroma free hemoglobin (SFH) was determined in a Cannon-Ubbelohde Semi Micro Dilution Type viscometer at 37°C. In all cases the kinetic energy correction factor was small enough to be neglected. The diluent was physiological saline at pH 7.4. The concentrations of these solutions were in the range from 2.5 to 30gm/100 ml of Hgb. These data are shown graphically in Fig 1 along with a similar set of measurements on albumin solutions. It should be noted that below the 10gm/100ml level, there is very little difference between the relative viscosities (η_{rel}) of the two solutions. The viscosity of the SFH solutions could be quantitatively represented by the generalized Mooney equation.

$$\ln \eta_{rel} = \frac{[\eta]c}{1 - (k/c)\,[\eta]c} \qquad (1)$$

In equation 1, $[\eta]$ is the intrinsic viscosity in dl/g, c, the concentration (g/dl) and k/v is a parameter which takes into account the molecular asymmetry and "crowding". From our data, $[\eta]$ was found to be 0.045 dl/g and k/v was 0.38. Using these values, it was possible to represent the experimental data to better than 5% deviation.

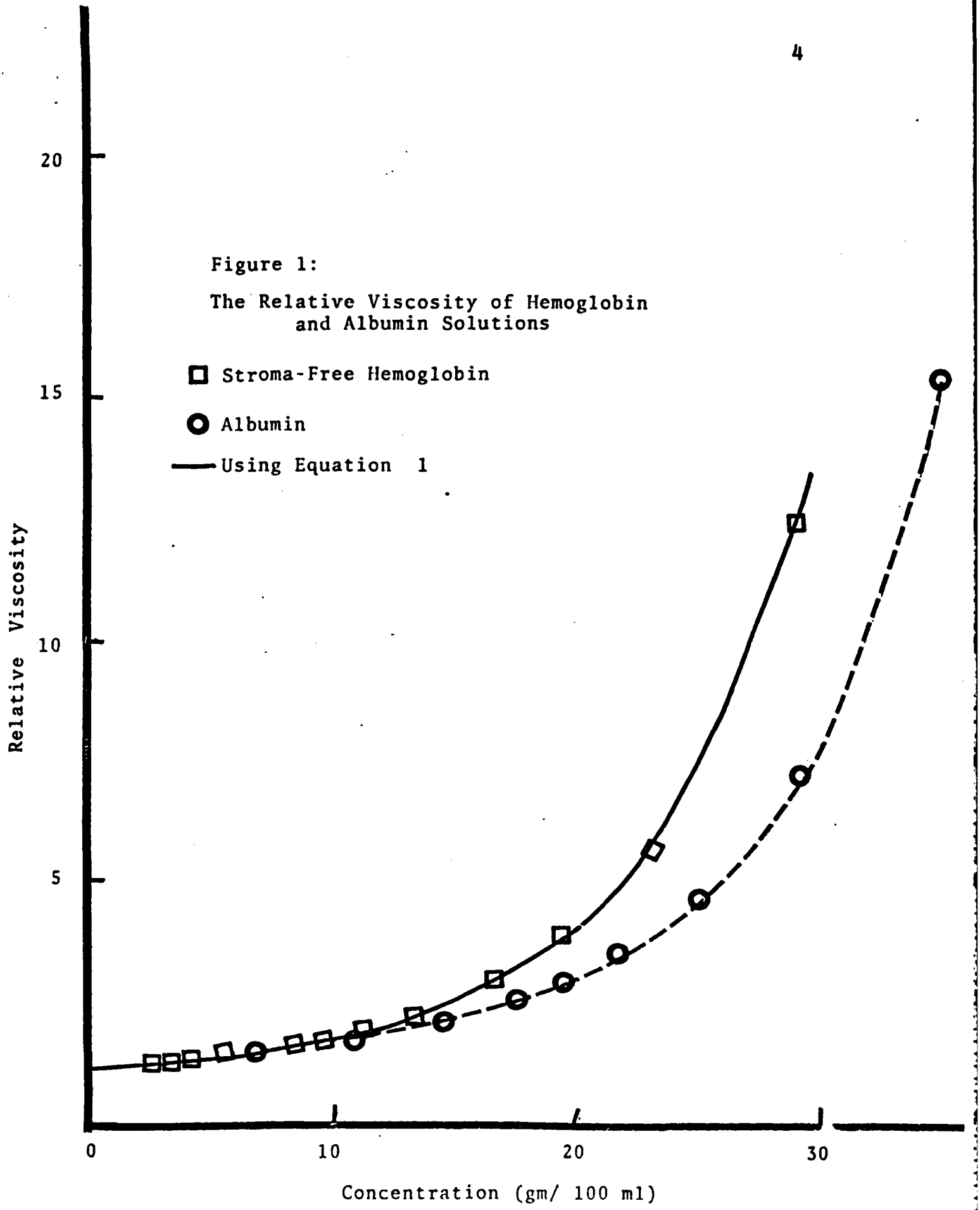


Figure 1:

The Relative Viscosity of Hemoglobin and Albumin Solutions

Simha showed that the intrinsic viscosity of a disc-like particle could be represented by

$$[\eta] = \frac{16}{15} \quad \frac{f}{\tan^{-1} f} \qquad (2)$$

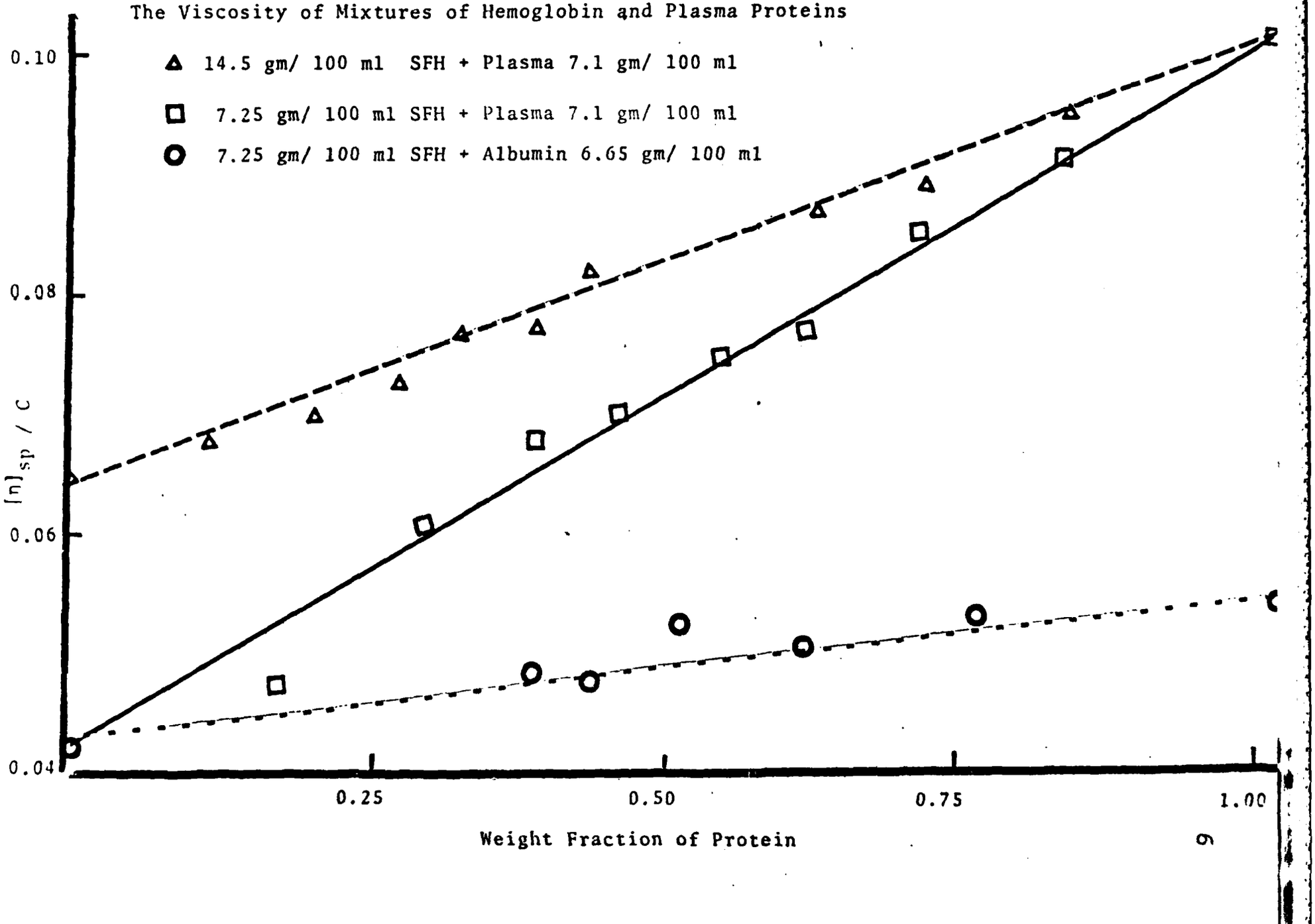
Where f was the axial ratio. If one uses the values given by Tanford, f can be taken as 2.1 to 3.4, yielding an intrinsic viscosity between 0.0347 and 0.0493 dl/g which agrees well with our experimentally determined value.

To examine the effects of mixtures of SFH and plasma protein, viscosity measurements were made using two different SFH starting concentrations; one of 14.5 gm/100ml and the other 7.25 gm/100ml. These data are shown in Fig 2 where the specific viscosity, η_{sp}, divided by the concentration is graphed versus the weight fraction of plasma protein. The specific viscosity is used because it represents the contribution to the viscosity due to the amount of polymer (SFH and/or protein) present above that of the solvent. The figure indicates a linear relationship. For comparison, mixtures of SFH (7.25 gm/100ml) and albumin (6.5gm/100ml) are also graphed in Fig 2.

The effect of shear rate on the viscosity of SFH has not been definitely resolved. At high concentrations (i.e. above 15%), shear thinning may be a controlling factor in the microcirculation. Some

FIGURE 2 :

The Viscosity of Mixtures of Hemoglobin and Plasma Proteins



of these measurements are shown in Fig 3A and B where the viscosity (centipoise) is graphed versus rate of shear (sec^{-1}).

The non-Newtonian flow behavior of several mixtures of whole blood and SFH was examined. In Fig 4, the viscosity at four different hematocrits is graphed versus the shear rate with a 6.1% SFH solution as the diluent. Below 30% Hct, the flow is Newtonian and independent of the shear. The effect of hemodilution is quite apparent and has a marked effect on the viscosity reduction. A similar experiment is illustrated in Fig 5. In this investigation, the diluent is 14.5% SFH solution. The non-Newtonian flow region even extends to the 17.7% Hct mixture. This is in part due to the non-Newtonian behavior of this concentration of SFH which is indicated in Fig 3. The hemodilution effect is not as pronounced as with the 6.1% SFH solution as illustrated with the samples of 52 and 42.4% Hct.

In addition to the flow properties, the rate of oxygen transport is of equal importance for cardiovascular and respiratory activity. As an indication of this property, it is normal to express the ratio of the hematocrit to the viscosity (Hct/η) as a function of the hematocrit (Hct) at a fixed shear rate. This is shown in Fig 6 for whole blood diluted with 6.1% SFH solution at the shear rates of 46 sec^{-1} and 23 sec^{-1}. These shear rates were chosen because they illustrate the greatest effect of this activity. The graph indicates that a maximum does exist at a hematocrit of about

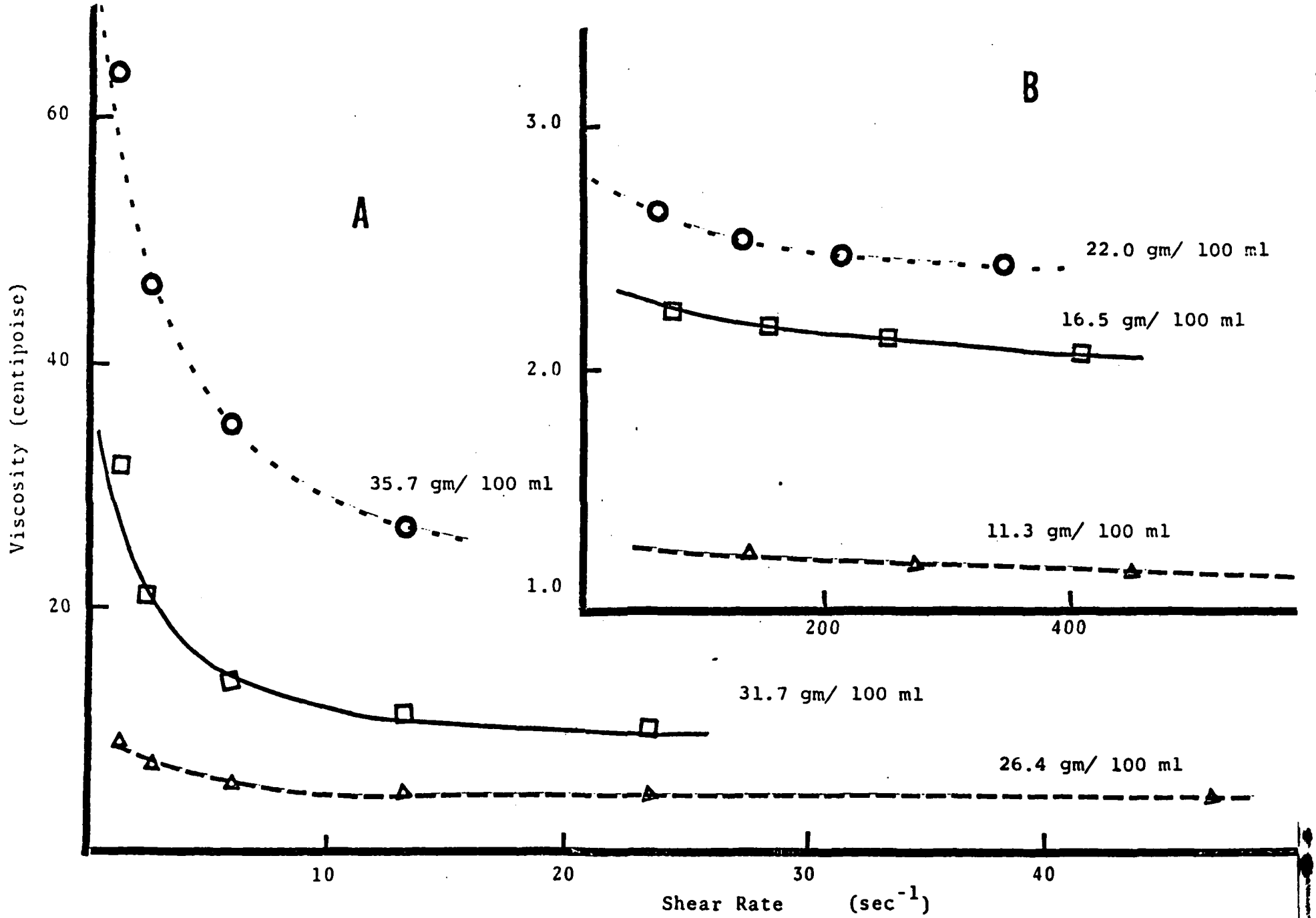


FIG 3: The Effect of Shear Rate on the Viscosity of Hemoglobin Solutions

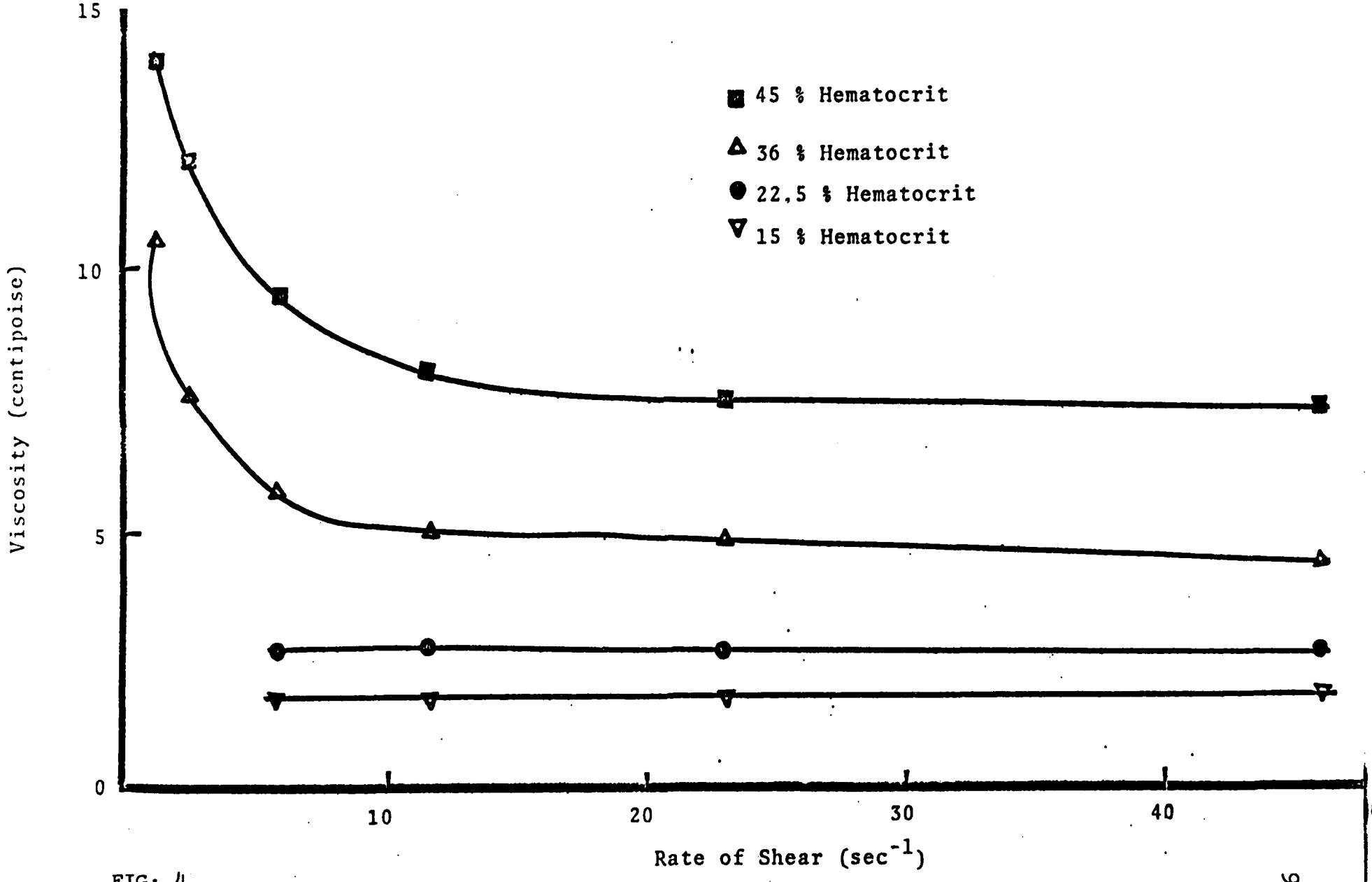

15
10
5
0
Viscosity (centipoise)
45 % Hematocrit
36 % Hematocrit
22,5 % Hematocrit
15 % Hematocrit
10
20
30
40
Rate of Shear (sec-1)

FIG: 4

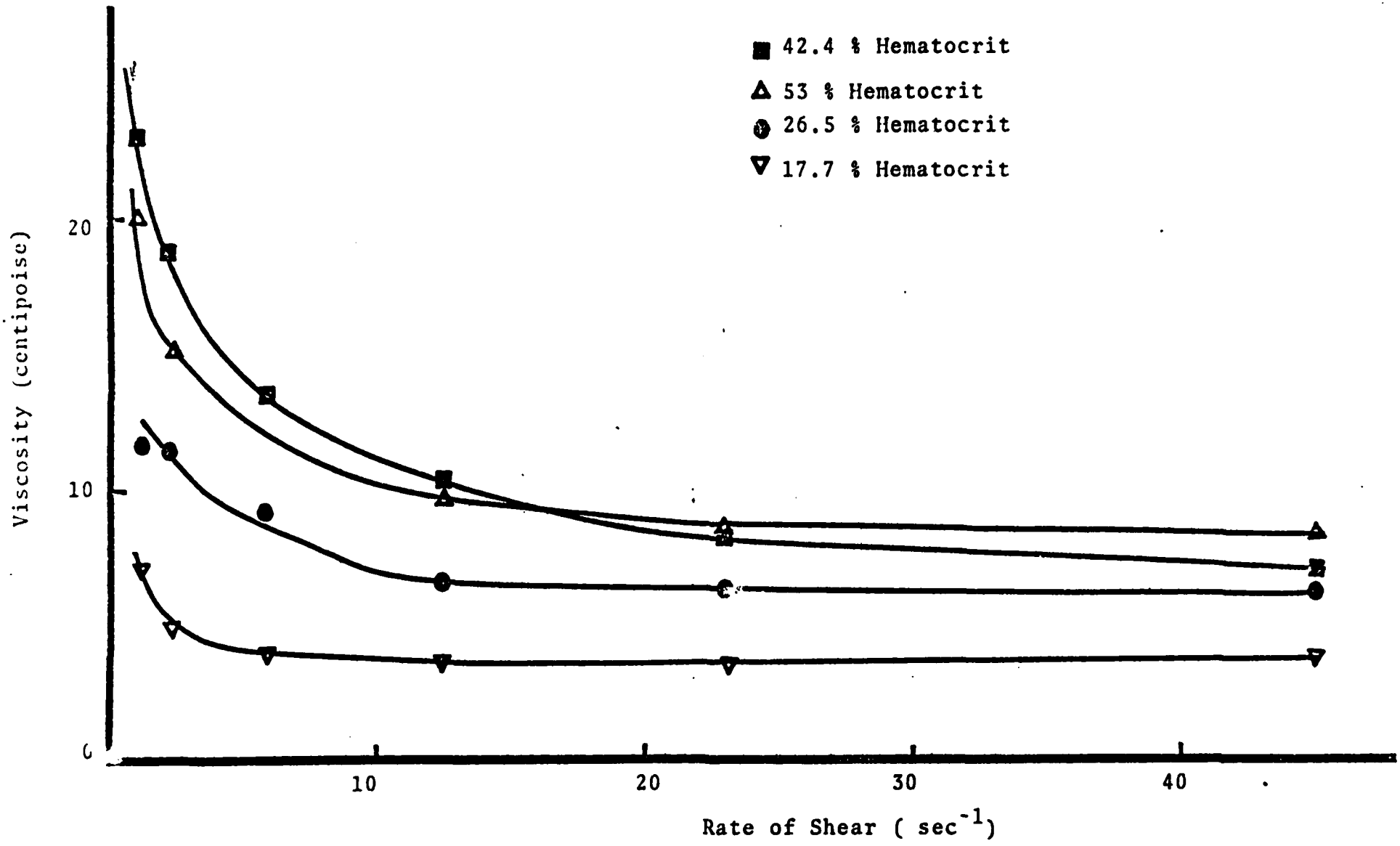

42.4 % Hematocrit
53 % Hematocrit
26.5 % Hematocrit
17.7 % Hematocrit
Viscosity (centipoise)
20
10
0
10
20
30
40
Rate of Shear (sec^{-1})

FIG: 5

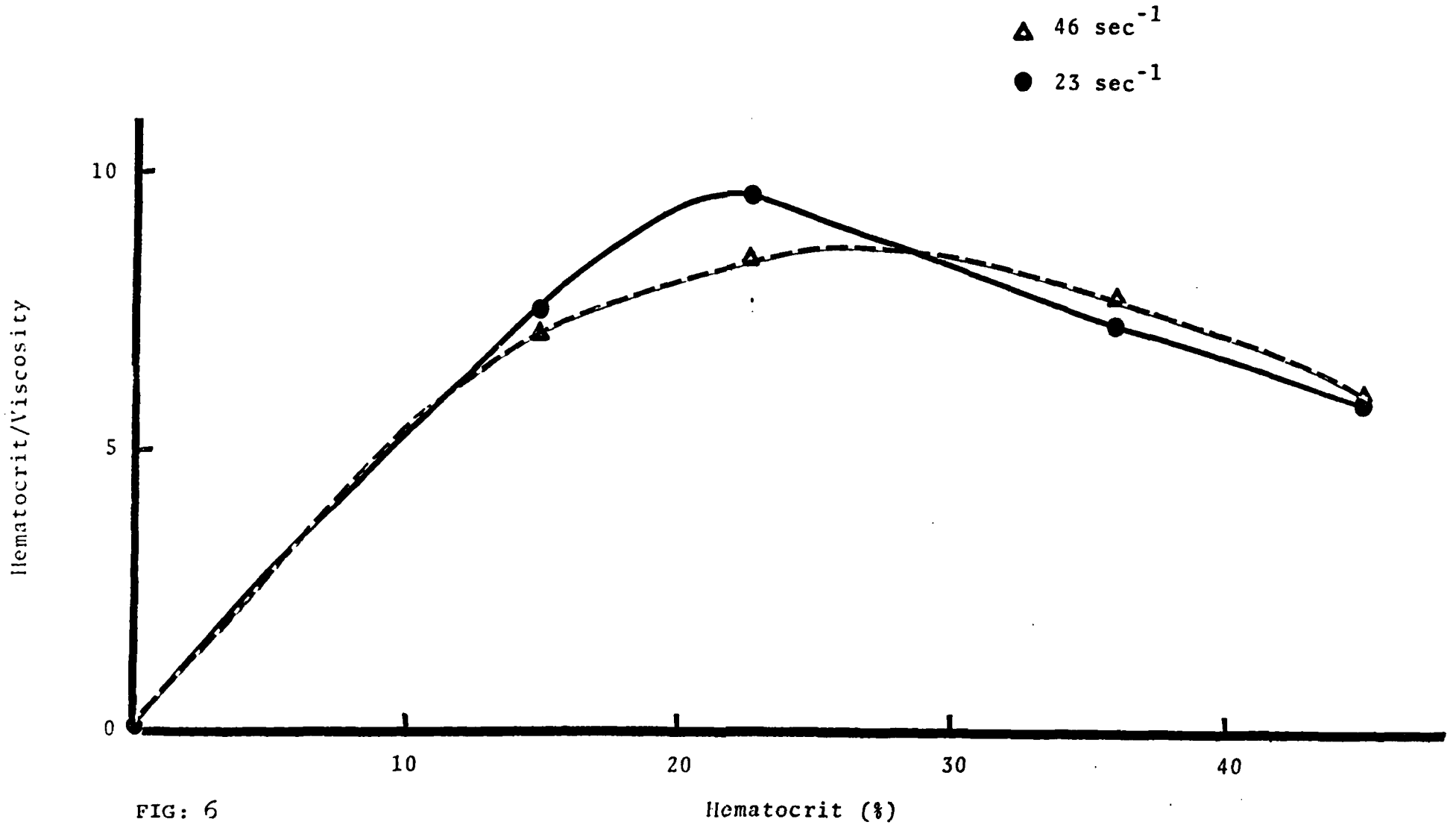
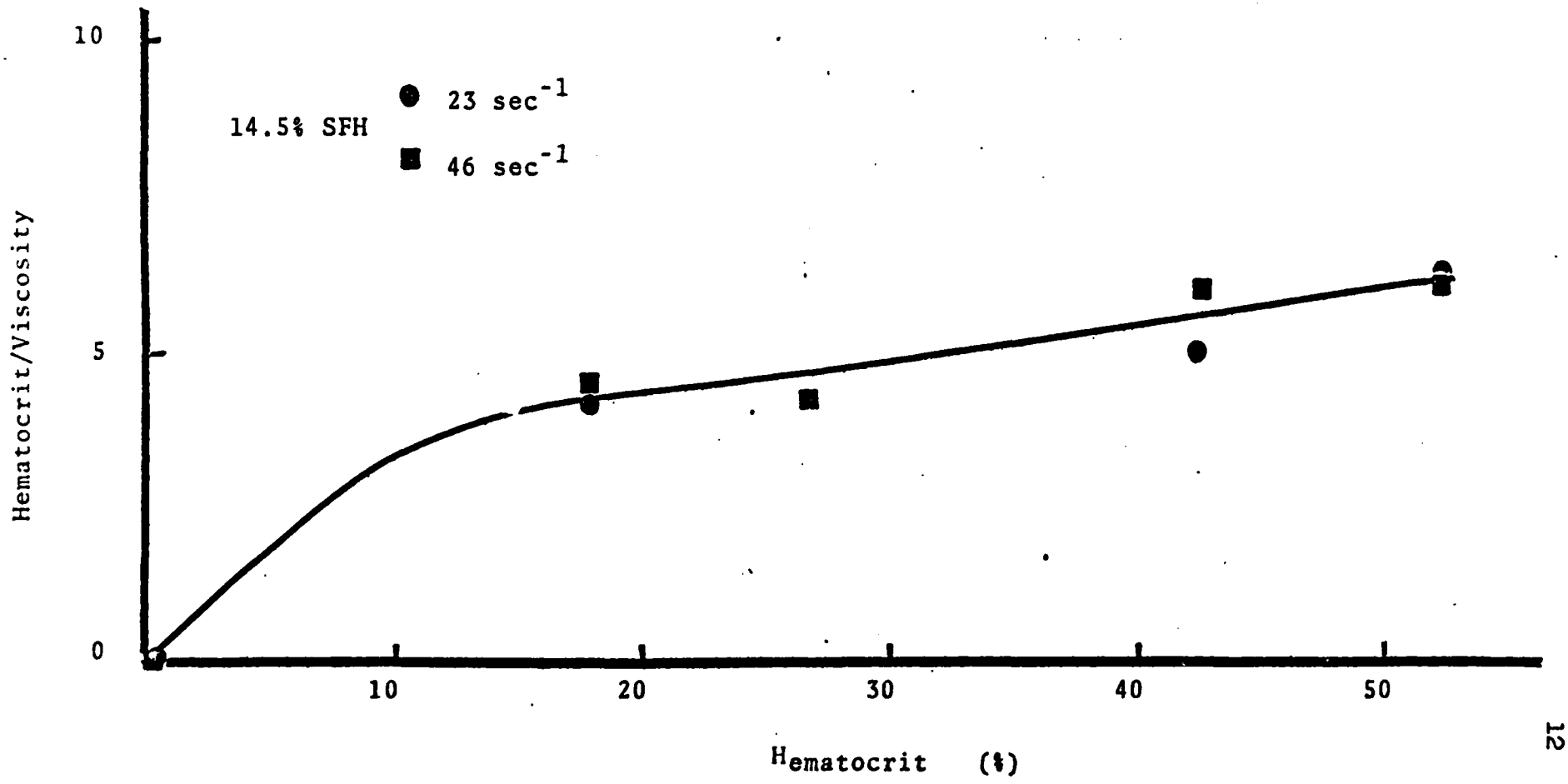

46 sec-1
23 sec-1
10
5
0
Hematocrit/Viscosity
10
20
30
40
Hematocrit (%)

FIG: 6

FIG: 7


10
5
0
14.5% SFH
23 sec-1
46 sec-1
Hematocrit/Viscosity
10
20
30
40
50
Hematocrit (%)

25%. Similar data are shown in Fig 7. However in this graph, the diluent was 14.5% SFH solution. It is important to observe that the overall effect of this activity is reduced, there is no maximum in the curve and the effect is almost constant and independent of hematocrit. This indicates that the oxygen transport is apparently not directly increased with increasing SFH concentration. This is a complex relationship and intimately involved with the flow patterns in Fig 4 and 5.

Technical Objective (2). The examination of the concentration dependence of the osmotic pressure is mixtures of SFH at various hematocrits and plasma proteins.

The measurement of the colloidal osmotic pressure was determined with a modified Zweifach micro-osmometer. A constant temperature bath was used to control the temperature to better than $\pm 0.5^{\circ}C$. The concentration range extended to about 14 gm/100ml. It was shown that the data could be represented by

$$\left(\frac{\pi}{c}\right)^{1/2} = \left(\frac{\pi}{c}\right)_o^{1/2} \left[1 + \Gamma 2 \frac{c}{2}\right] \qquad (3)$$

In this equation, π represents the osmotic pressure at concentration C, and $\Gamma 2$ is the second virial coefficient which is a measure of the polymer-solvent interactions. A graph of $\left(\frac{\pi}{c}\right)^{1/2}$ vs.C results in a straight line. From the intercept, $\left(\frac{\pi}{c}\right)_o$ 1/2 one obtains the number average molecular weight, Mn.

$$\left(\frac{\pi}{C}\right)_o = \frac{RT}{Mn} \qquad (4)$$

From the slope, one evaluates $\Gamma 2$,

$$\Gamma 2 = \frac{2\,(\text{slope})}{\left(\frac{\pi}{C}\right)_o^{1/2}} \qquad (5)$$

Equation (3) is used for the osmotic pressure rather than a plot of (π/C) vs. C, because it eliminates the curvature which may lead to erroneous extrapolation procedures.

The osmotic pressure data are summarized in Table 1 for three different temperatures. Also included are the osmotic pressure data for other commonly used plasma expanders, hydroxyethyl starch (HES), dextran, polyvinylpyrrolidone (PVP) and albumin. The values of $\Gamma 2/[\eta]$ are presented because they should remain constant for a particular polymer series.

It is interesting to note the magnitude of the $\Gamma 2$ values for SFH in comparison to the series of other plasma expanders. The relative size indicates that the SFH solutions are approaching the ideal polymer solution, $\Gamma 2 = 0$.

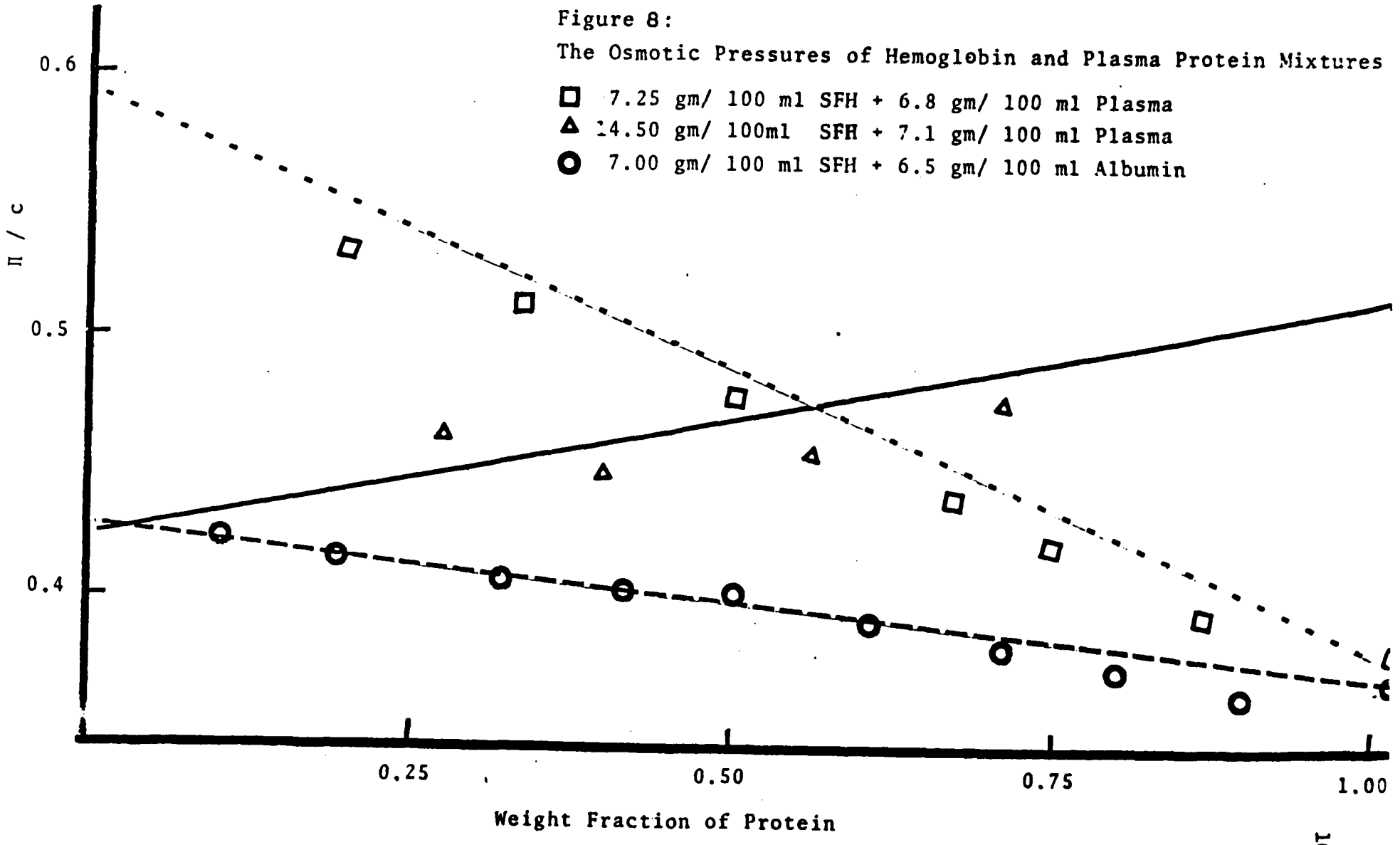
Some data of mixtures with SFH and plasma proteins are shown in Fig 8. In this graph the osmotic press/concentration (π/C) is graphed versus the weight fraction of plasma. Two SFH concentrations are presented, 7.25 gm/100ml and 14.5 gm/100ml. For comparison, some data with 7.0 gm/100ml SFH and 6.5 gm/100ml albumin mixtures are

Table 1

The Osmotic Pressure and Intrinsic Viscosity of Stroma-Free Hemoglobin and Some Plasma Substitutes

Substance	M_n	Γ_2 $(\frac{ml}{gm})$	$[\eta]$ $(\frac{dl}{gm})$	$\Gamma_2/[\eta]$
		Temperature 37°c		
SFH	69,200	3.59	0.0385	93.2
Albumin	81,000	7.01	0.0380	185.0
		Temperature 30°c		
SFH	67,300	3.49	0.0350	99.7
Dextran-Rheomacrodex	33,400	12.4	0.175	70.9
Dextran-Macrodex	49,100	20.2	0.275	73.3
PVP-Plasadone	23,700	8.68	0.202	43.0
Hydroxyethyl-Starch	81,500	21.2	0.181	117
		Temperature 20°c		
SFH	66,800	3.21	0.0320	100.3

Figure 8:
The Osmotic Pressures of Hemoglobin and Plasma Protein Mixtures



also graphed.

The osmotic pressure of mixtures of whole blood at different concentrations of SFH also present an interesting situation. These data are shown in Fig 9. In this figure, the osmotic pressure in cm. of H_2O is graphed against the volume percent of SFH in the mixture. These determinations were made at four SFH concentrations, 3.5%, 7.0%, 9.5% and 15.2%. The values of the blood hematocrits are given in parenthesis on the graph. It is interesting to observe the maxima that occurs at the concentrations of 7.0% and greater at about 75 volume % of SFH. This would suggest that during infusion with SFH solutions the vessels in the circulatory system are in a hyper osmotic state relative to whole blood. This may have certain advantages in the treatment of shock.

Technical Objective (3). The quantitation of the erythrocyte sedimentation rate (ESR) as a function of Hct, SFH and plasma protein concentration.

The erythrocyte sedimentation rate (ESR) studies were made in the automatic sedimentimeter. This unit not only follows the settling process but it also electronically generates the first derivative of the curve, that is, the true rate of settling as a function of time. This is a new concept in ESR and has the potential of becoming a diagnostic indicator. A typical set of data are shown in Fig 10 where both the settling curves and the first derivatives are taken directly from the sedimentimeter. A summary of data is

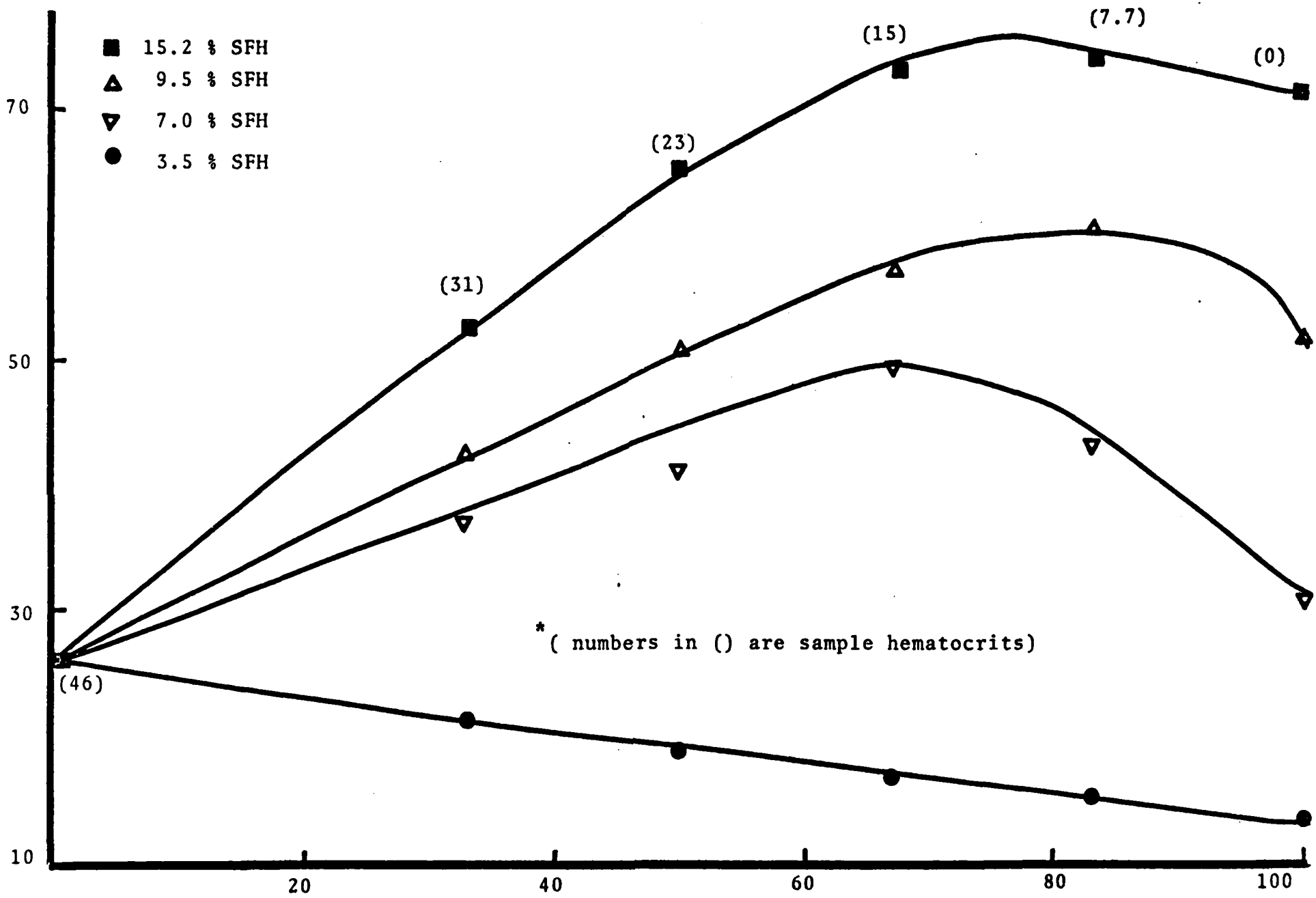

15.2 % SFH
9.5 % SFH
7.0 % SFH
3.5 % SFH
(46)
(31)
(23)
(15)
(7.7)
(0)
70
50
30
10
20
40
60
80
100
*(numbers in () are sample hematocrits)
Volume Percent SFH

FIG: 9

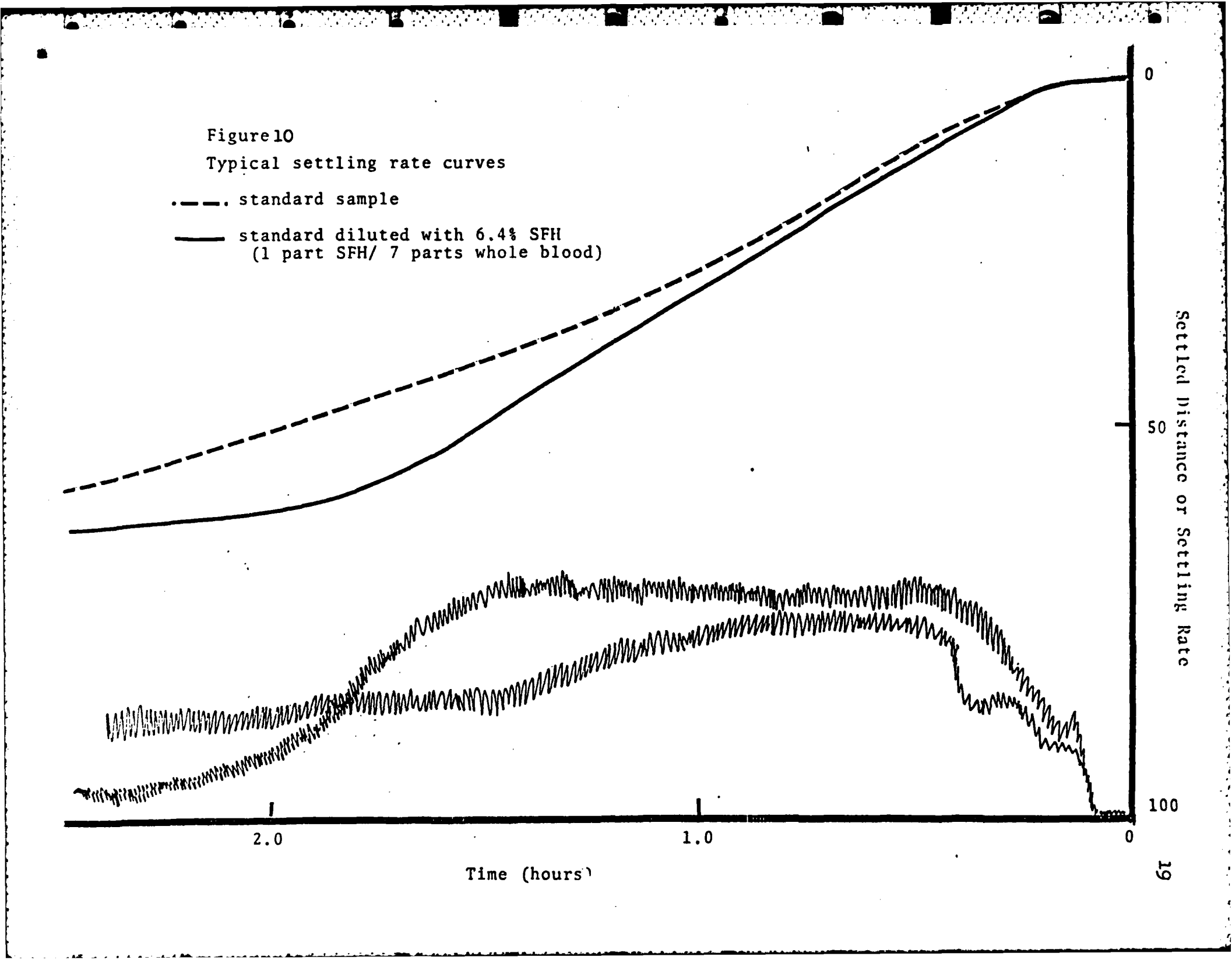


Figure 10

Typical settling rate curves

.—.—. standard sample

——— standard diluted with 6.4% SFH
(1 part SFH/ 7 parts whole blood)

presented in Table 2. It is our contention that the maximum velocity, V_{max} (cm/hr), is the most meaningful single parameter to document the ESR.

A careful examination of Table 2 indicates some interesting results. First, it appears that a total replacement of the plasma with either a 7% SFH solution or a 6.5% albumin solution eliminates all settling. This could be interpreted to mean that the forces which aggregate the red cells causing the settling, have been eliminated. To compare a commonly used plasma expander with SFH two samples of hydroxyethyl starch were used (High MW=450,000; Low MW=264,000). Both samples over a wide range of concentration greatly increase the ESR.

The interesting feature relates to the whole blood case with different volume ratios of 6% SFH solution. These ratios are typical of those used in infusion procedures. It is observed that there is only a small change in the ESR over this clinically significant range of whole blood-SFH mixtures.

Technical Objective (4). The determination of the effect of SFH on red cell membrane properties using the techniques of hemolytic malonamide kinetics, osmotic fragility and microsieving.

We have shown that the technique of hemolytic malonamide kinetics offers a means of examining the properties of the red cell membrane. This method is particulary convenient and accurate for dilute solutions of SFH, that is, a 7% solution. The optics of the

Table 2

The Effects of Replacement Fluids on the Erythrocyte Sedimentation Rate

	System	Hct	Vmax($\frac{cm}{hr}$)
A	1) Whole Blood	41	3.67
	2) Washed cells in 7% SFH	41	no settling
B	1) Whole Blood	36	7.98
	2) Washed cells in 50% plasma+50% 7% SFH	36	1.77
	3) Washed cells in 50% plasma+50% 0.9% Saline	36	1.01
C	1) Whole Blood	42	11.08
	2) Washed cells in 66% plasma-34% 10% SFH	42	4.62
D	1) Whole Blood	42.5	4.69
	2) Washed cells in 6.5% Albumin	42.5	no settling
E	Washed Cells in		
	1) 1% High MW HES	38	1.01
	2) 2% High MW HES	38	6.46
	3) 3% High MW HES	38	18.37
	4) 4% High MW HES	38	26.98
F	Washed Cells in		
	1) 2% Low MW HES	37	0.63
	2) 4% Low MW HES	41	6.21
	3) 6% Low MW HES	38	10.39
G	Washed Cells in		
	1) 6% Low MW HES+0% SFH	36	11.15
	2) 6% Low MW HES+0.606% SFH	36.5	5.32
	3) 6% Low MW HES+0.303% SFH	37	8.11
H	Whole Blood	38	6.78
	1) 6% SFH-Whole Blood (1v:6v)	31.5	5.95
	2) 6% SFH-Whole Blood (1v:7v)	32	5.95
	3) 6% SFH-Whole Blood (1v:9v)	34	5.95

spectrophotometer place a limit on this technique. In this investigation, we were concerned with the effect of overnite incubation of whole blood and 7% SFH mixtures. Previously we indicated that the kinetics could be followed by the single mathematical expression

$$\text{Percent Hemolysis} = \frac{1}{1 + \exp\left[\beta(t-t_{50})\right]} \qquad (6)$$

In equation (6), t represents the time t_{50} is the half life of the reaction and β is a parameter measuring the breadth of the red cell kinetic activity. In Fig 11, we have a sample of whole blood as the control. In Fig 12.13 and 14 are presented data using 25% 50% and 75% by volume of 7% SFH solution whole blood mixtures.

The values of the t_{50} and β are given on each figure. There does not appear to be any significant change in the hemolytic kinetics caused by the presence of the SFH solution.

The kinetic hemolysis described above measures the ability of the red cell membrane to withstand a slowly increasing osmotic pressure (30 minute time period). It also provides information on whether or not the SFH present interfers with the transport of relatively small molecules (i.e. malonamide) across the erythrocyte membrane.

In order to measure the effect of the presence of SFH on the red cells response to a rapidly increasing osmotic pressure (1 to 2 minute time period) data were taken using a Kalmedic Fragilograph. This instrument provides simultaneously cummulative and derivative hemolysis data as a function of decreasing extracellular sodium

FIG: 11

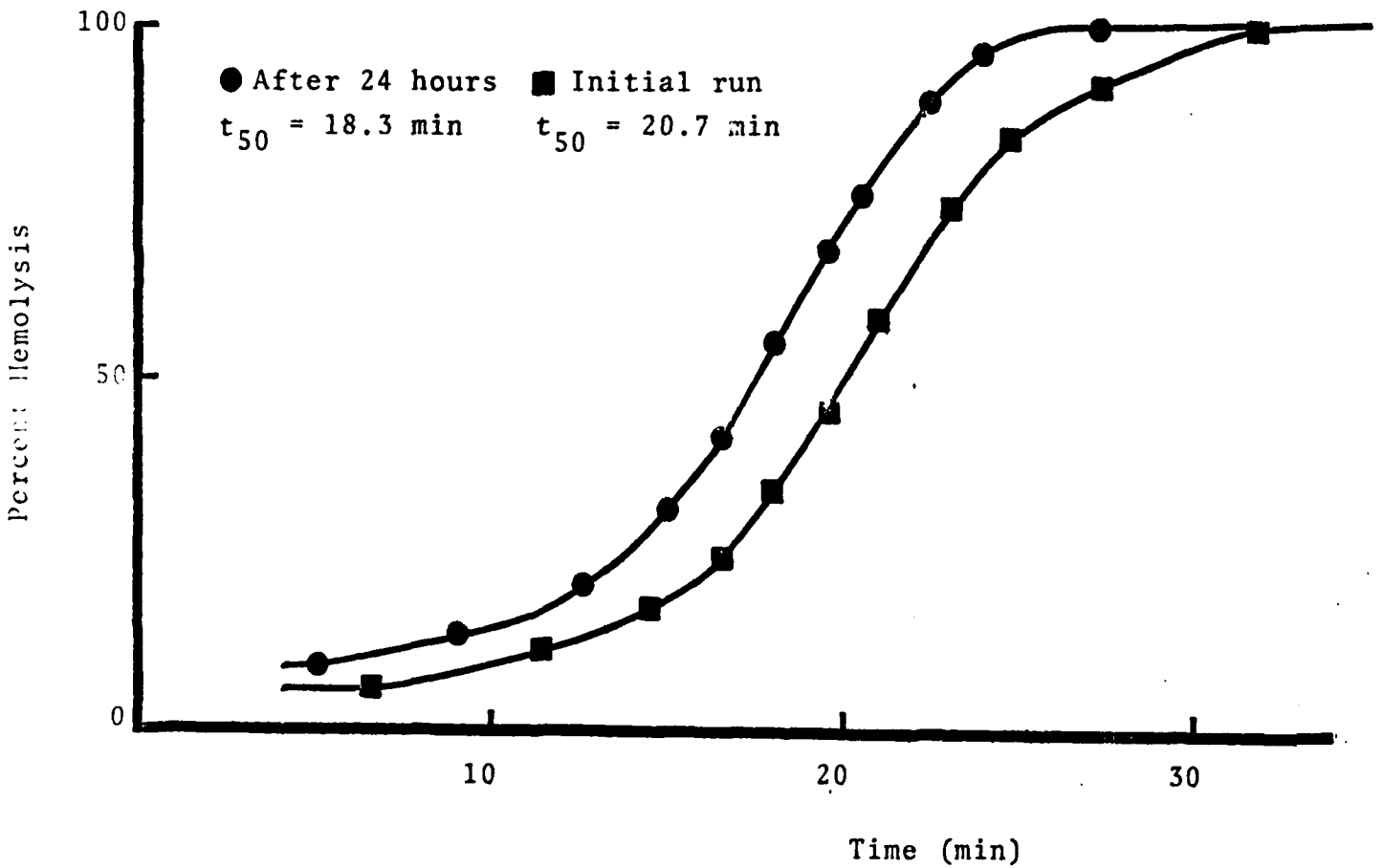

100
50
0
● After 24 hours
■ Initial run
t_{50} = 18.3 min
t_{50} = 20.7 min
Percent Hemolysis
10
20
30
Time (min)

FIG: 12

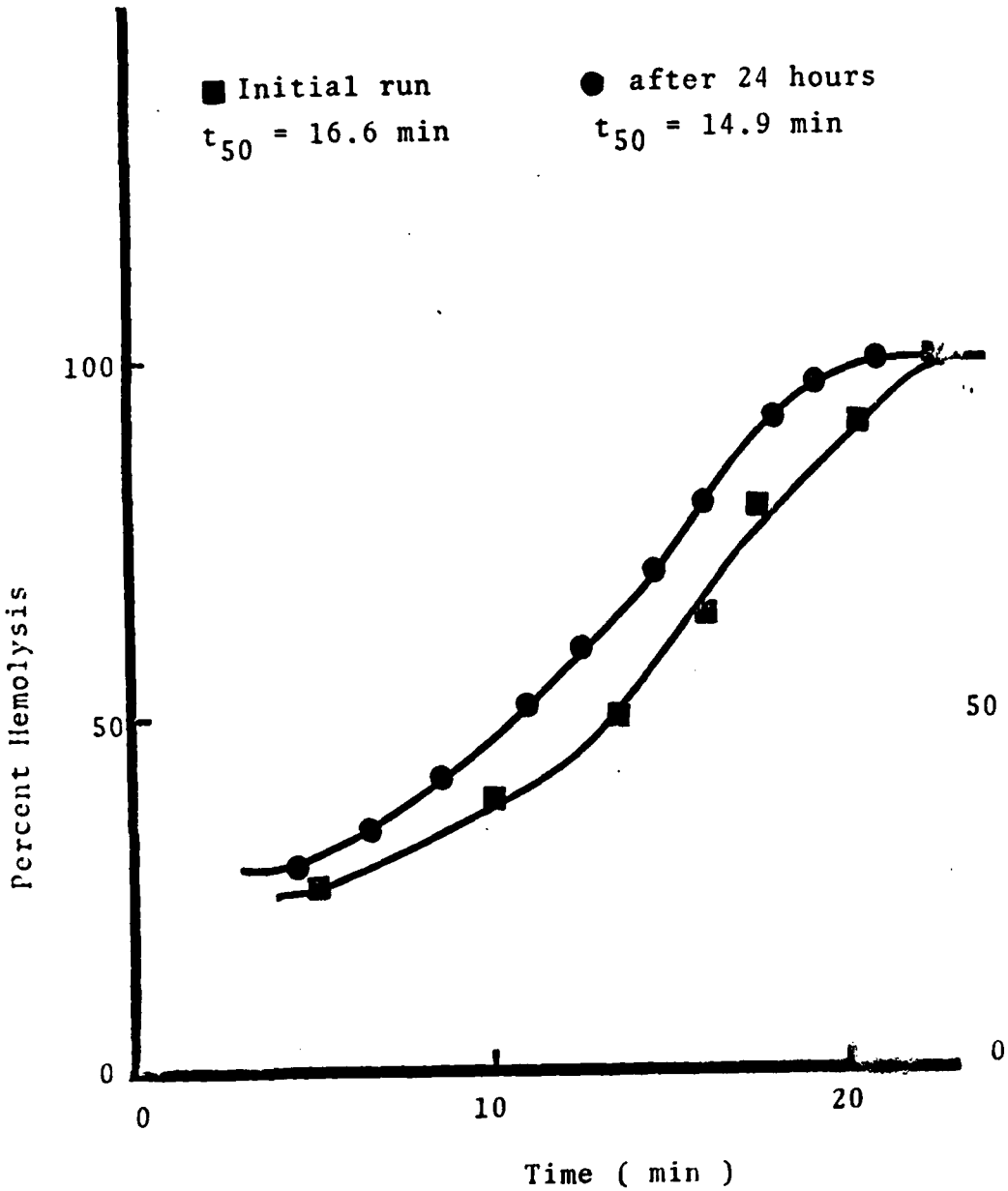
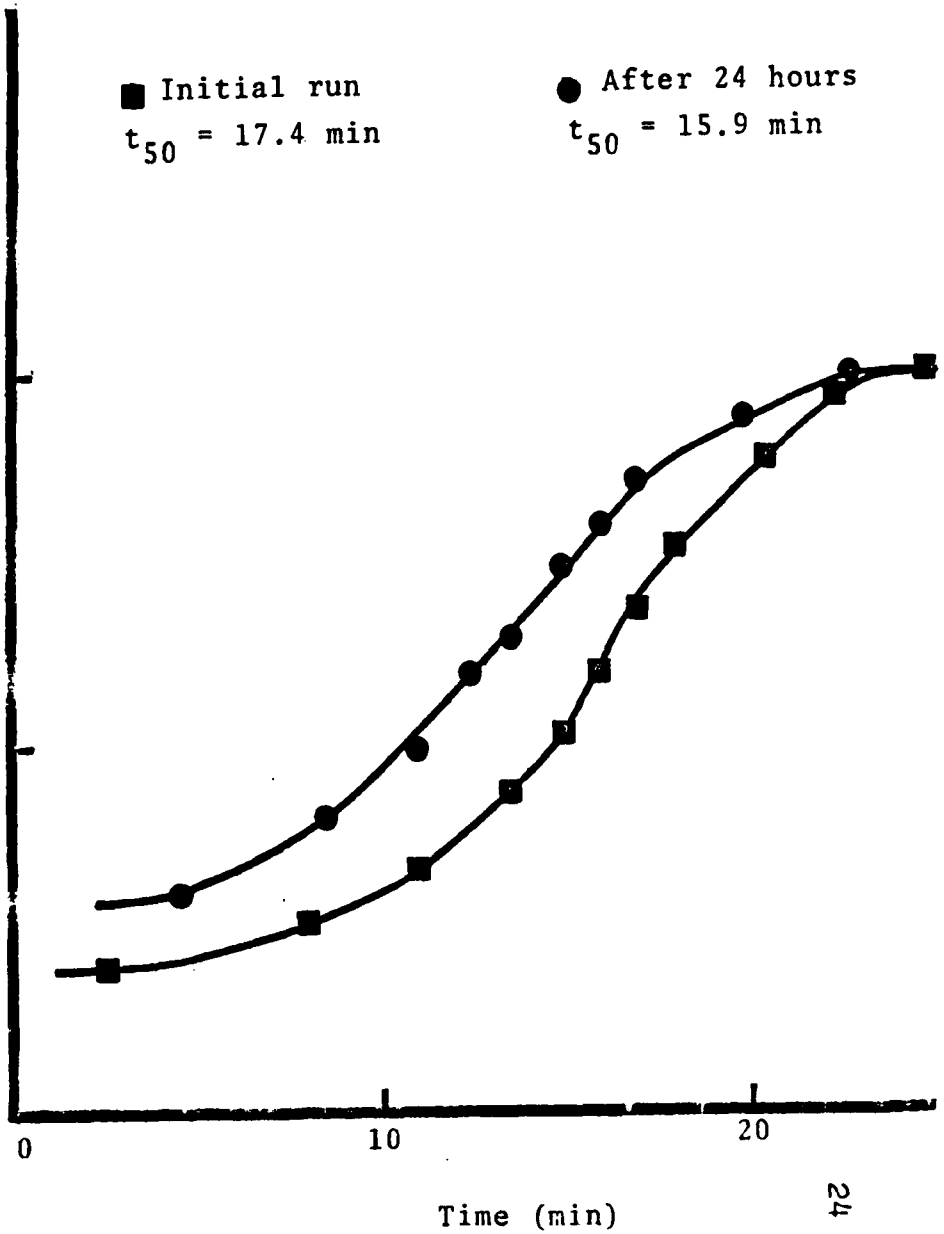

■ Initial run
t_{50} = 16.6 min
● after 24 hours
t_{50} = 14.9 min
100
50
0
Percent Hemolysis
0
10
20
Time (min)

FIG: 13


■ Initial run
t_{50} = 17.4 min
● After 24 hours
t_{50} = 15.9 min
50
0
0
10
20
Time (min)

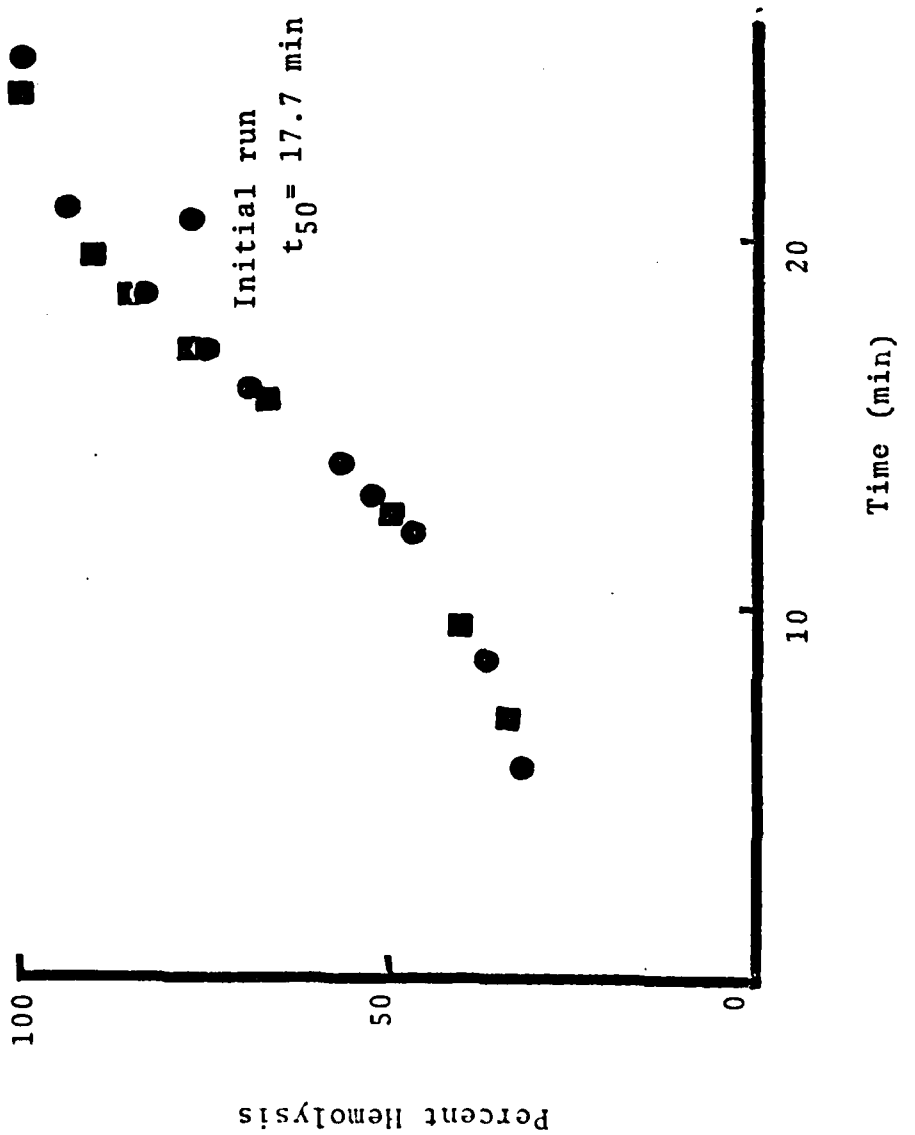

Percent Hemolysis
100
50
0
10
20
Time (min)
Initial run
t_{50}= 17.7 min
After 24 hours
t_{50}= 18.2 min

FIG: 14

chloride concentration. These data give the mean fragility of the red cell population. The skewness of the derivative curve immediately indicates when a particular segment of the population is being preferentially attacked by a hemolytic agent. All measurements were made at 37°C using a sodium chloride-sodium veranol buffer at pH=7.4. Three experimental conditions were used. In the first instance the fragility of a mixture of 7% SFH with whole blood (50/50 by volume) was compared with a sample of the whole blood. Both of the samples were incubated at 4°C and the measurements repeated after 4 hours. Typical data can be seen in figs 15 and 16. The presence of the SFH resulted in a slight (7 to 8%) increase in fragility. The extra cellular saline concentration at which half the red cell population had hemolyzed, C_{50}, is listed on the graph for comparison.

The second series of experiments repeated the above protocal except that the samples were kept at room temperature (22°C) rather than 4°C. Representative curves are shown in figs 17 and 18 as can be seen from the C_{50} values listed on the figure, the presence of SFH does not alter the hemolysis behavior of the erythrocytes.

Neither of the above procedures showed a significant difference in the hemolysis in the presence or absence of SFH. Similiar experiments identical to those above except that the hemoglobin solution was not mixed with the whole blood until immediately before the fragility determination also showed no difference among the various samples.

FIG: 15

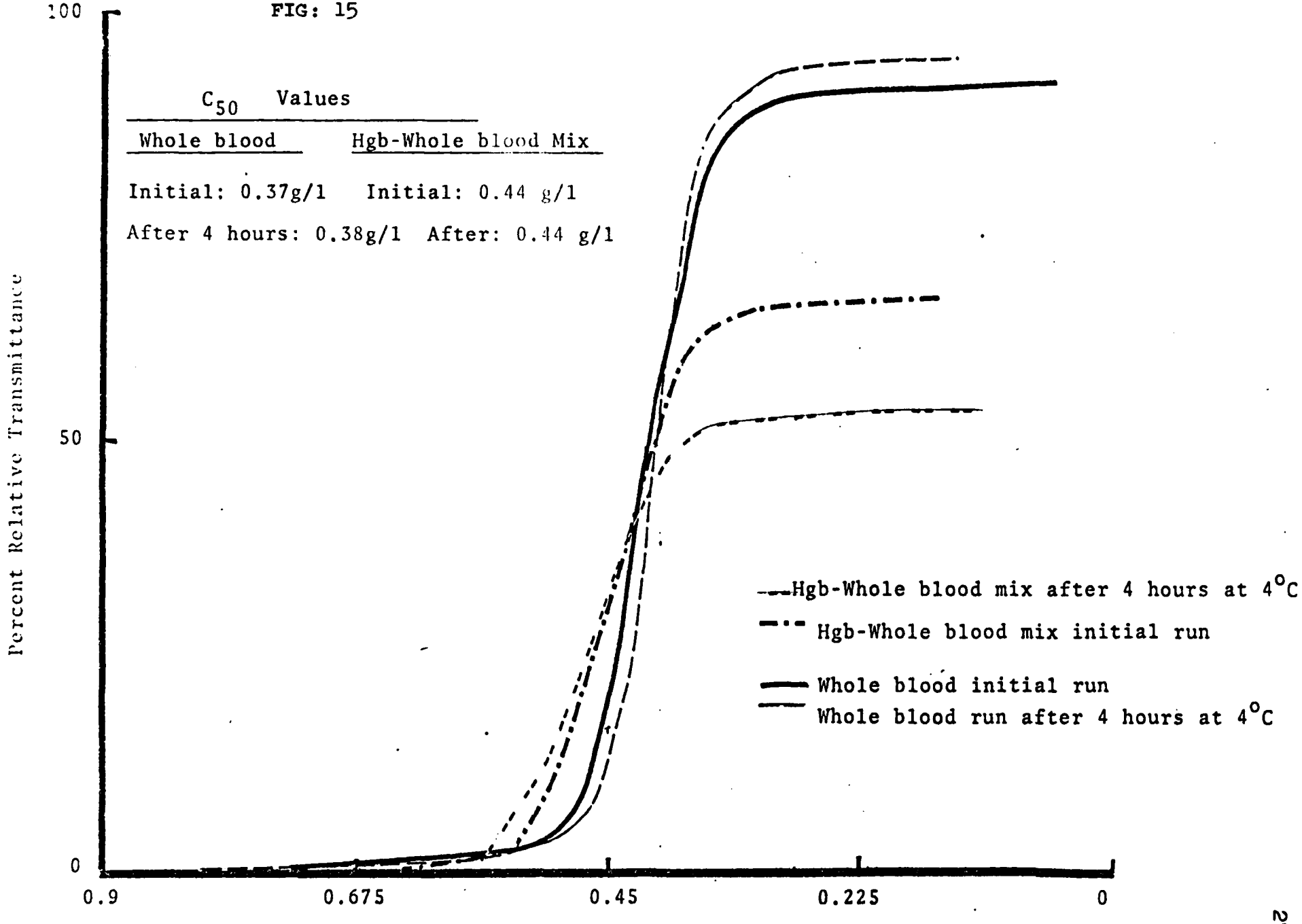
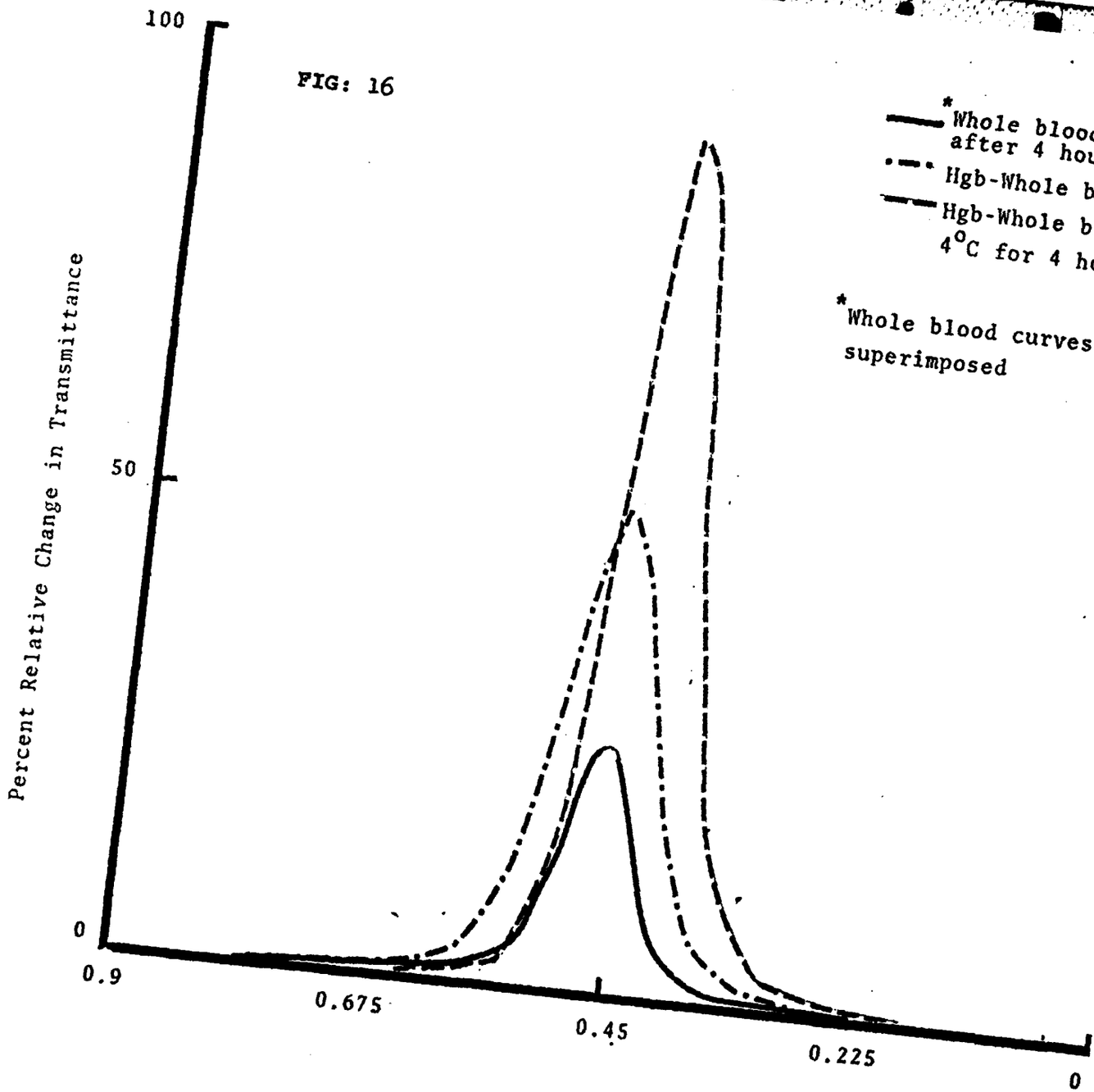

100
50
0
Percent Relative Transmittance
0.9
0.675
0.45
0.225
0
C_{50} Values
Whole blood
Hgb-Whole blood Mix
Initial: 0.37g/1
Initial: 0.44 g/1
After 4 hours: 0.38g/1
After: 0.44 g/1
Hgb-Whole blood mix after 4 hours at 4°C
Hgb-Whole blood mix initial run
Whole blood initial run
Whole blood run after 4 hours at 4°C

FIG: 16



*Whole blood initial run and repeat after 4 hours/ samples at 4°C before run

Hgb-Whole blood mix (50/50) initial run

Hgb-Whole blood mix after storage at 4°C for 4 hours

*Whole blood curves were indistinguishly superimposed

FIG: 17

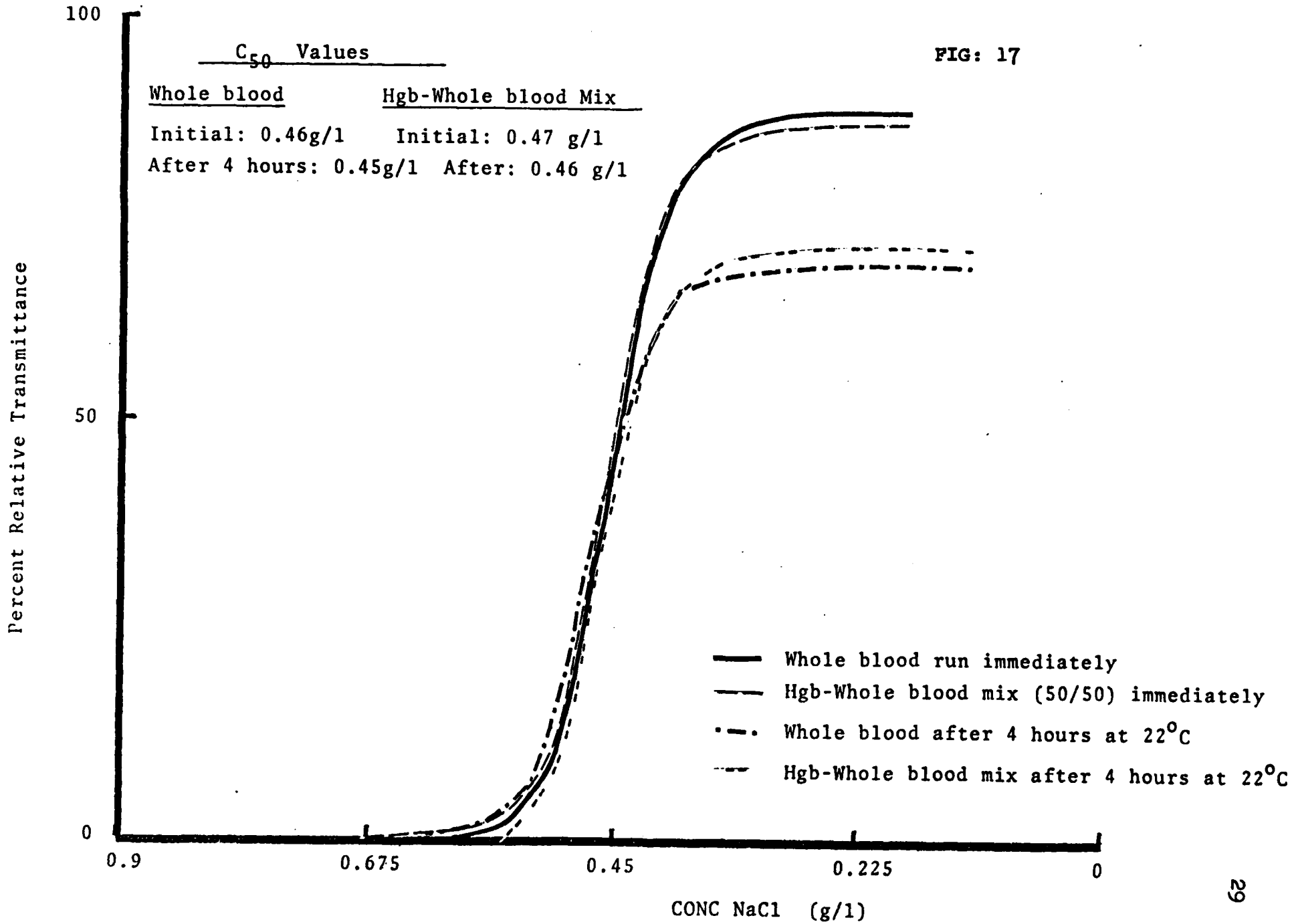
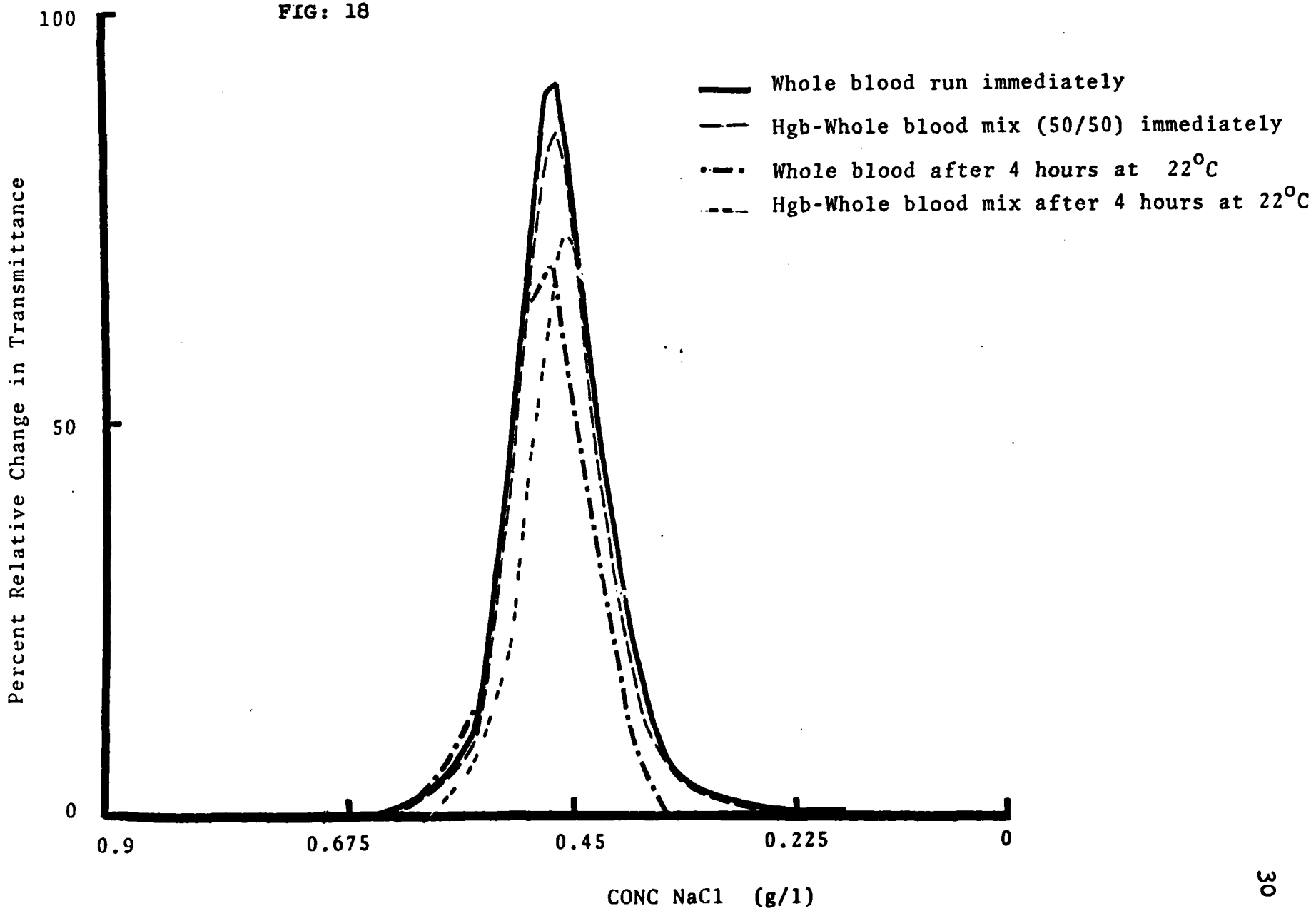

100
50
0
Percent Relative Transmittance
C_{50} Values
Whole blood
Initial: 0.46g/1
After 4 hours: 0.45g/1
Hgb-Whole blood Mix
Initial: 0.47 g/1
After: 0.46 g/1
Whole blood run immediately
Hgb-Whole blood mix (50/50) immediately
Whole blood after 4 hours at 22°C
Hgb-Whole blood mix after 4 hours at 22°C
0.9
0.675
0.45
0.225
0
CONC NaCl (g/1)

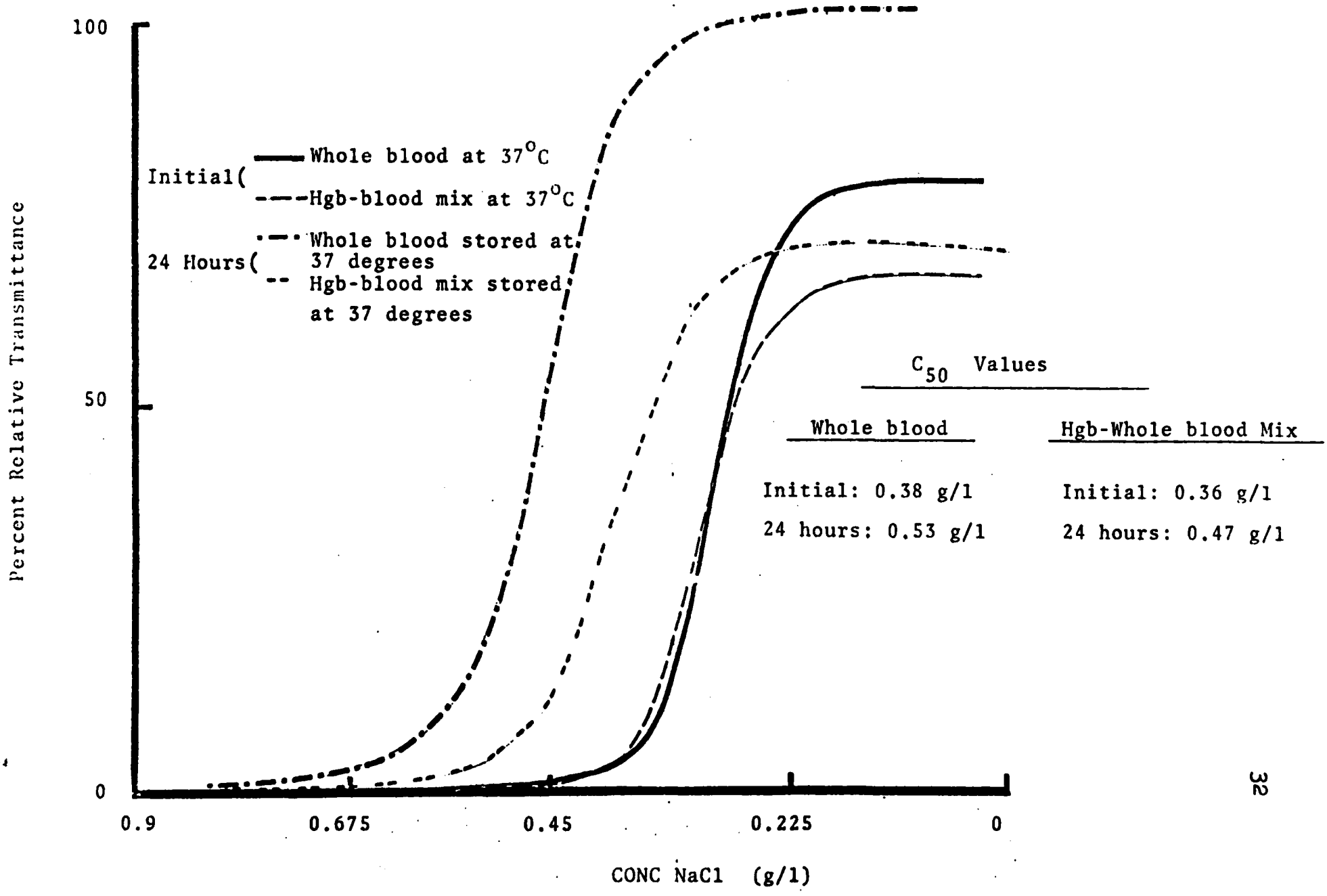
FIG: 18


100
50
0
Percent Relative Change in Transmittance
0.9
0.675
0.45
0.225
0
CONC NaCl (g/l)
Whole blood run immediately
Hgb-Whole blood mix (50/50) immediately
Whole blood after 4 hours at 22°C
Hgb-Whole blood mix after 4 hours at 22°C

The last data set compared initial fragility values with those from samples incubated for 24 hours at 37°C. An average data set is shown in figs 19 and 20. It is most easily seen from fig 20 that initially there is no discernable difference between the whole blood and the Hgb whole blood mixture. After incubating for 24 hours both samples showed an increased fragility. It should also be noted that both derivative curves are broadened but remain very symmetrical. This indicates that no given part of the population (say, only the older erythrocytes) are being affected more by the treatment or the SFH. Careful attention should be given to the relative positions of the derivative curves. The SFH mixture seems to offer significant protection to the red cells during the incubation. The addition of SFH does not prevent the increase in fragility noted for the whole blood sample but it does reduce this increased fragility. This is best seen quantitatively by comparing the C_{50} values listed on fig 19.

Both of the above experimental procedures indicate that the addition of SFH does not increase the suceptibility of the red cell to osmotic fragility and may offer a slight but significant degree of protection.

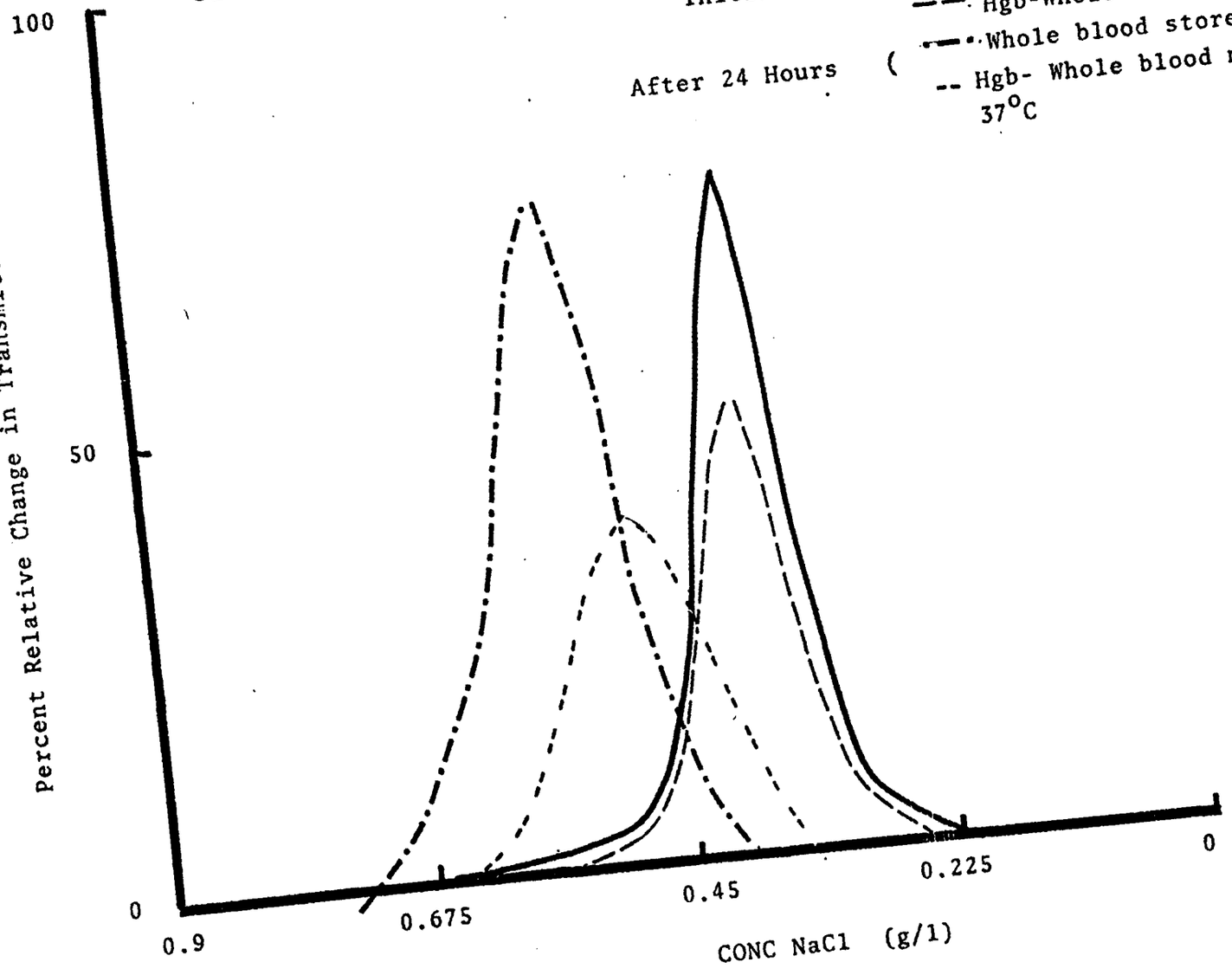
FIG: 19



C_{50} Values

Whole blood	Hgb-Whole blood Mix
Initial: 0.38 g/1	Initial: 0.36 g/1
24 hours: 0.53 g/1	24 hours: 0.47 g/1

FIG. 20


Initial run (
Whole blood at 37°C
Hgb-Whole blood mix (50/50) 37°C
After 24 Hours (
Whole blood stored at 37°C
Hgb- Whole blood mix stored at 37°C
Percent Relative Change in Transmittance
100
50
0
0.9
0.675
0.45
0.225
0
CONC NaCl (g/l)

Conclusions: The preliminary results of this study indicate that the biophysical characteristics of SFH make it an extremely suitable blood substitute. One of the key factors in this conclusion is its availability from out dated whole blood. At the concentration that is currently being considered as an infusing solution, 7%, it is a Newtonian fluid having a viscosity very similar to plasma. At this concentration, SFH also has very favorable hemodilution properties. This is a feature which is a critical factor for any plasma substitute. However the added advantage here is the potential oxygen transport ability which most expanders lack. There also appears to be little interaction between the plasma proteins and SFH in the concentration range between 7 and 14% as indicated from viscosity measurements. However at concentrations exceeding 10%, SFH solutions exhibit a slight non-Newtonian flow pattern that may present some complications in the microcirculation and distract from its advantage as a hemo diluent.

The osmotic behavior of SFH solutions indicate that it approaches an ideal polymer-solvent system as illustrated by the small value of the second virial coefficient, especially when compared to other plasma substitutes including dextran, hydroxyethyl starch and polyvinyl pyrrolidone. In mixtures with whole blood at a series of hematocrits, SFH solutions at concentrations between 7 and 15.2% all appear to be hyper osmotic. This biophysical property could certainly have significant ramifications in cases of severe shock.

Another notable feature of SFH solutions is its ability to maintain the integrity of whole blood and not to initiate aggregation of the erythrocytes. A common technique which illustrates this phenomena is the ESR. With some plasma expanders, such as dextran and polyvinyl pyrrolidone, there is a noticeable increased ESR. Our studies with HES, both the high and low molecular weight types, also indicate an increased ESR. The investigations of the ESR in this study with SFH, either alone with whole blood or in combination with the low molecular weight HES, indicate the potential usefulness of SFH in maintaining stability in these systems. The fact that SFH does not promote erythrocyte aggregation is another advantage that it may have as an infusion fluid for the treatment of shock.

In the presence of SFH solutions, the transport properties of the red cell membrane are apparently not effectively altered. This has been illustrated in the hemolytic malonamide kinetic studies. The neglible changes in the half life values for this kinetic process over a twenty four hour period substantiate this fact. To further explore the erythrocyte membrane properties, similar studies of the osmotic fragility were performed. Although the results are preliminary, it should be stressed that SFH solutions may offer protection against hemolysis when used as an transfusion solution.

Recommendations: Stroma free hemoglobin solution may be the ideal blood substitute. It meets many of the stringent requirements demanded by a transfusing agent. The investigations presented here substantiate this belief. Additional studies on SFH solutions should include the following:

1) An in depth examination of the protective action of SFH solutions on blood in hemolytic anemias, muscular dystrophy and other red cell membrane abnormalities;
2) An investigation of the improvement of the oxygen transport properties with mixtures of SFH solutions and blood from sickle cell disease and thalassemia. These genetic blood disorders lack adequate organ oxygenation promoting local hypoxia and acidosis;
3) Study the metabolic activity of SFH mixtures and whole blood as measured by heat production with the technique of microcalorimetry;
4) Improve the dwell times of SFH by:
    a) Forming a compound with hydroxythyl starch;
    b) Encapsulating the SFH;
    c) Synthesizing a polyhemoglobin.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